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Rab31 is expressed in neural progenitor cells and plays a role in their differentiation

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ABSTRACT

Rab31 is expressed in both GFAP- and nestin- positive fibres in regions of neurogenic potential in the adult mouse brain. To investigate the role of Rab31 in neural progenitor cells (NPCs), we cultured NPCs and found significant levels of Rab31 expression in these cells. Rab31 levels showed a sharp initial decrease and then reappeared gradually in a subpopulation of astrocytes when NPCs were induced to differentiate. Silencing of Rab31 hindered, while overexpression enhanced, the differentiation of NPCs to astrocytes. Our results suggest a previously unrecognised role for Rab31 in influencing the differentiation and fate of NPCs.

1. Introduction

In the embryonic mammalian brain, radial glia (RG) develop into the various progenitor cells that give rise to the three main cell types of the brain [1]. RG are positive for both nestin and glial fibrillary acidic protein (GFAP), markers for progenitor and astrocytic cells respectively [2]. While RGs are not found in the adult brain, it is believed that as the animal matures, a population of RGs retain their neurogenic potential and become the neural progenitor cells (NPCs) of the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, the two main areas of neurogenesis in the adult [3]. These nestin- and GFAP-positive cells [4] are known as Type B astrocytic cells in the SVZ [5] and radial astrocytes in the SGZ [6], and are believed to be only a subset of the adult astrocytic population in this region [7,8]. When examined by electron microscopy, these cells have the same ultrastructural characteristics as typical mature astrocytes [9,10]. They are believed to give rise to new neurons, and possibly also the other glial cell types [11] during adult neurogenesis.

Non-progenitor, mature astrocytes exist in the rest of the brain parenchyma. These astrocytes, the characteristically star-shaped glial cells, are GFAP-positive but lack nestin. They also express S100β, a calcium binding protein [12]. Astrocytes, among other things, provide metabolic support for neurons, and maintain the blood–brain barrier. Co-culture analyses also show that astrocytes stimulate neurogenesis from stem cells isolated from the SVZ and SGZ. This ability might be a regional characteristic of these astrocytes, as astrocytes isolated from the spinal cord do not have the same effect when co-cultured [13,14].

The developing and adult nervous system house/host a complex network of trafficking processes, which include the cycling of synaptic vesicles, secretion and internalisation of signalling molecules and their receptors, and the migration of cells requiring the trafficking of adhesion molecules, etc [15–17]. As such, it is conceivable that proteins involved in intracellular trafficking would have a large impact on the functioning of the nervous system. One example is the Rab (Ras-related protein in brain) family of small GTPases. Rabs function as molecular switches and play critical roles in intracellular membrane transport [18]. Rabs interact with a variety of upstream regulatory proteins which serve to activate or deactivate them, as well as with effector proteins that act downstream. Specificity of Rab function is thus conferred by these proteins [19–21]. Rab5, for example, was shown to function in axonal and dendritic endocytosis [22], and mutations in Rabex5, the activator of Rab5, resulted in defects in release of presynaptic neurotransmitters [23]. Rabs have also been found in non-neuronal glial cells, but their roles in glia have been less well defined [24–26]. It is conceivable that in mature oligodendrocytes Rabs might

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play a role in regulating the process of myelination [27], which requires heavy membrane traffic. In astrocytes, vesicular trafficking is important, among other things, for the exocytosis of neuropeptides that regulate cerebral blood flow, and the regulation of glutamate transporter density at the plasma membrane, which helps to regulate the strength of synaptic signalling [28].

In our initial experiments detailing the expression profile of Rab31 in mouse tissues, Rab31 was found to be enriched in the brain [29]. Immunohistochemical analysis revealed that Rab31 could be found in GFAP-positive cells in the adult mouse brain, suggesting that Rab31 is expressed in astrocytes. In the embryonic mouse brain, Rab31 was seen in nestin-positive cells, indicative of radial glia. We have also previously shown that Rab31 participates in the endocytic trafficking of ligand-bound epidermal growth factor receptor (EGFR) [29]. Fine control of EGFR signalling is particularly important in the proliferation and differentiation of NPCs. In view of these results, we reasoned that a closer look at a possible physiological role of Rab31 in neurogenic areas of the brain would be warranted.

2. Materials and methods

2.1. Gene constructs

Mouse Rab31 cDNA was kindly provided by Prof Mitsunori Fukuda (Tohoku University, Japan). The cDNA was digested with *EcoRI* and *SalI* restriction enzymes (New England Biolabs) and inserted into the pDMyc expression vector, which is modified from pCI-Neo (Promega) to include the myc expression tag between the CMV promoter and the multiple cloning site. The resultant pDMyc-mouse Rab31 plasmid was used for overexpression of mouse Rab31 as described below.

2.2. Antibodies

Rabbit anti-serum against Rab31 was generated by immunisation with glutathione-S-transferase (GST) fused to the C-terminal 37 amino acids of Rab31 and used at 1:50 dilution. The following commercial primary antibodies were used at 1:200 dilution for immunofluorescence staining and 1:1000 dilution for Western blotting: βIII-tubulin (TuJ) (Research Diagnostics), 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (CNPase) (Abcam), GFAP (Sigma–Aldrich), nestin (Chemicon International), proliferating cell nuclear antigen (PCNA) (Abcam), doublecortin (DCX) (Santa Cruz Biotechnologies), EGFR (Merck Millipore), GM130 (BD Transduction Laboratories), SOX2 (Santa Cruz Biotechnologies) and γ-tubulin (Sigma). The following secondary antibodies were used for Western blotting at 1:5000 dilution: horse radish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies (All Eights Pte. Ltd., Singapore). The following secondary antibodies were used for immunofluorescence labeling at 1:100 dilution: fluorescein isothiocyanate (FITC), Texas-Red (TxR) or Cy5-conjugated anti-mouse, anti-rabbit, anti-sheep and anti-goat antibodies (All Eights Pte. Ltd., Singapore).

2.3. Primary mouse NPC culture

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore, and animals were handled in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. NPC isolation was performed with established protocols with modifications [30,31], and is described in more detail in the Supplementary material.

2.4. Transfection and RNAi-mediated silencing

Transfection of the purified pDMyc-mouse Rab31 expression vector for overexpression of Rab31 in NPCs was performed with XtremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocols.

siRNA (Integrated DNA Technologies, Inc)-mediated silencing in NPCs was carried out by Lipofectamine RNAiMAX (Invitrogen)-mediated transfection, according to the manufacturer's protocols. Transfection reagent was typically removed after 6 h, and analysis or follow-up experiments were performed after 48 h.

Rab31 siRNAs are based on the following sequences:

siRNA-1 5′-rGrGrArGrTrArCrGrCrTrGrArArTrCrCrArTrArGrGrTrGCC-3′
siRNA-2 5′-rGrTrArCrTrArCrCrGrArGrGrArTrCrTrGrCrTrGrCrArGCC-3′

Scrambled siRNA is based on the following sequence:

5′-rCrUrUrCrCrUrCrUrCrUrUrUrCrUrCrUrCrCrCrUrUrGrUGA-3′

2.5. Retroviral transduction

Engineered murine retroviruses were made to express GFP along with the shRNA. GFP expression was under the control of the EF1α promoter, and shRNA against Rab31 was co-expressed under the control of the human U6 promoter in the same vector. shRNA was based on the following sequence: 5′-rArGrT rArCrG rCrTrG rArArT rCrCrA rTrArG rG-3′. Retroviruses were obtained as previously described [32]. Further information can be found in the Supplementary material.

2.6. Reverse-transcription (RT) and real-time polymerase chain reaction (PCR)

To determine successful silencing of Rab31, total RNA was harvested from cells using Qiagen RNA isolation kit (Qiagen, Singapore). One-step RT-PCR was performed using Qiagen RT-PCR kit (Qiagen) and products were run on a 3% agarose gel and visualised.

Changes in levels of mRNA were also analysed as NPCs differentiate. Total RNA was extracted from approximately 2×10^6 cells at 0, 4, 8, 24, 48 and 120 h after differentiation. Real-time PCR with SYBR green detection (Applied Biosystems) was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Detailed methods and sequences of primers used can be found in the Supplementary material.

2.7. Western blot

For Western immunoblot analysis, tissue from Sprague–Dawley rat or cells from NPC cultures were lysed with lysis buffer (50 mM Tris pH8, 1 mM EDTA pH8, 150 mM NaCl, and 1% Triton X-100 with protease inhibitor cocktail (Roche)) for 60 min. Lysates were then subjected to reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), typically with 7% or 13% gels, electroblotted onto Hybond C-extra (Amersham Biosciences, UK) nitrocellulose membranes, and probed with the respective antibodies in 3% bovine serum albumin (BSA) in PBS-T (0.05% Tween-20 in phosphate buffered solution (PBS)).

2.8. Immunostaining and confocal microscopy

For immunohistochemistry, 4% paraformaldehyde-perfused C57/BL6 mice brain tissue was imbedded in optimal cutting temperature (OCT) medium (Electron Microscopy Sciences) and sec-

tioned by cryostat at 20 μm thickness (Leica Microsystems). For immunocytochemistry, cells plated on cover slips and subjected to various treatments were fixed with 4% paraformaldehyde. After sequential incubation with the primary and secondary antibodies, fluorescence labelling was visualised using a Carl Zeiss 710 confocal imaging system. Further information can be found in the Supplementary material.

2.9. Quantitative and statistical analysis

Quantitative analysis of the percentage of cells positive for the marker of interest was performed for the images obtained from confocal microscopy. Three fields of view per coverslip were captured as images for quantification. Labelled cells were considered positive for expression of their respective markers if the fluorescence exceeded at least twice the background level, as measured by Carl Zeiss' Zen 2010 image analysis software. Data and error bars shown in bar graphs represent means and standard error of means (S.E.M.) of 3 independent experiments each assayed from 3 individually seeded coverslips. At least 100 cells were examined in total. Statistical analysis was performed using unpaired Student's *t*-test.

3. Results

3.1. Rab31 expression in the neurogenic regions of the adult rodent brain

The presence of Rab31 in radial glia [29] prompted us to investigate whether Rab31 could also be found in neurogenic regions of the adult rodent brain. We have previously shown that Rab31 is found in GFAP-positive astrocytes throughout the adult mouse brain [29]. Here, immunostaining of the adult mouse brain revealed Rab31-positive cells in two regions which house neural progenitor cells, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal region (Fig. 1A). In a crude dissection of the rat brain to obtain tissue lysate for Western blotting from various subregions, we also found Rab31 expressed at high levels in the hippocampus (Fig. 1B). Closer inspection of the hippocampal region of the adult mouse brain revealed an abundance of Rab31-positive cells in both the dentate gyrus (DG) and the CA1 region (Fig. 1C). Astrocytes in the CA1 region are likely to be normal, mature parenchymal astrocytes typically with a characteristic "star" shape [12], while neural progenitor cells are believed to reside in the SGZ, with radial projections into the granular layer [9]. We observed that the Rab31-positive cells in the CA1 region were indeed of the typical star shape, similar to those found in the cortical region (Fig. 1Cii and iii) while Rab31-positive cells in the DG could exhibit either astrocytic or radial glia morphologies (Fig. 1Ci). Fibres of these Rab31-positive cells are found to extend from the SGZ into the granular layer. These fibres are not TuJ positive (and are therefore not neurites) (Fig. 1Di), but were positive for nestin, a marker for neural progenitors (Fig. 1Dii), EGFR (Fig. 1Diii) and (sex determining region Y)-box 2 (SOX2), a marker for pluripotent progenitor cells (Fig. 1Div). The Rab31-positive cells do not colocalise with doublecortin (DCX), a marker for immature neurons that develop from the progenitor cells (Fig. 1Dv). The morphology and marker phenotype suggests that these Rab31-positive cells may be undifferentiated neural progenitor cells. Dividing neural progenitor cells are believed to express EGFR [33,34]. The presence of nestin, EGFR and SOX2 in GFAP-positive, Rab31-positive cells suggests that these might indeed be neural progenitor cells, or the radial astrocytes, of the SGZ [6]. It is likely that the Rab31-positive cells observed in the SVZ are of a similar nature.

3.2. Rab31 in undifferentiated neural progenitor cells

Having seen that Rab31 is expressed in the neurogenic areas of the adult mouse brain, we asked what roles Rab31 might play in neural progenitor cells. To investigate this, we used cultured neural progenitor cells (NPCs) harvested from E15 mice. Such NPCs are multipotent, EGF-responsive, and can be induced to differentiate in various media that favour either the differentiation to astrocytes or neurons, respectively [30,31].

We found Rab31 to be expressed in the undifferentiated NPCs in the perinuclear region (colocalising with the Golgi marker GM130) in most, if not all, cells. This Rab31-positive staining was specific, as it was obliterated when cells were transfected with Rab31 siRNA (Fig. 2A). These NPCs were also positive for both the progenitor cell-specific intermediate filament protein Nestin (Fig. 2Bi), proliferating cell nuclear antigen (PCNA) (Fig. 2Bii), have moderate levels of EGFR (Fig. 2Biii) and are SOX2-positive (Fig. 2Biv). These latter characteristics are in accordance with those of typical undifferentiated NPCs.

3.3. Rab31 in differentiated neural progenitor cells

To begin delineating a role for Rab31 in NPCs, we asked if Rab31 levels change as NPCs are induced to differentiate. Quantitative real-time PCR analysis of Rab31 mRNA transcripts as cells differentiated showed that Rab31 levels dropped transiently, but then increased, as NPCs differentiated to astrocytes, along with increasing GFAP levels (Fig. 3A). The changes seen were corroborated by immunofluorescence microscopy observations (Fig. 3B). These observations suggest that Rab31 expression may be linked to the differentiation of NPCs.

We observed that a subset of the GFAP-positive astrocytes obtained after differentiation were strongly immunopositive for Rab31. To further investigate this, we allowed the NPCs to differentiate under two different conditions, one that promotes a greater degree of astrocytic differentiation and the other that promotes a greater degree of neuronal differentiation. In both conditions, we observed that Rab31 is found in elevated levels in a subset of the GFAP-positive population, but not in TuJ-positve cells examined (Fig. 4A). The Rab31-positive cells also do not express doublecortin (DCX), a marker for immature neurons (Fig. 4B), or CNPase, a marker for oligodendrocytes (Fig. 4C). We also noted that these cells were now negative for PCNA and EGFR (Fig. 4D), indicating that these cells were no longer dividing progenitors. We found that only a subset of the total GFAP-positive population expresses Rab31 (Fig. 4E), as compared to undifferentiated NPCs where the perinuclear Rab31 is seen in most, if not all, cells. These observations indicate that Rab31 is expressed in NPCs, but its expression diminishes as the NPC population differentiates. Its expression is then re-established when NPCs differentiate into astrocytes, but not when NPCs differentiate into neurons or other cell types. These observations are consistent with our previous observations in vivo that Rab31 expression is most prominent in radial glial and adult astrocytes [29].

3.4. Effect of Rab31 silencing and overexpression on differentiation of neural progenitor cells

Given the ubiquitous presence of Rab31 in undifferentiated NPC and its initial diminishment as cells differentiate, Rab31 may help to maintain NPCs in an undifferentiated form. Alternatively, the subsequent increase in Rab31 levels in a subset of astrocytic cells also suggests that Rab31 may be a cell fate determinant for the differentiation of NPCs towards astrocytes instead of neurons. To investigate the first possibility, we silenced Rab31 expression in undifferentiated NPCs by retroviral transduction (Supp. Fig. 2A),

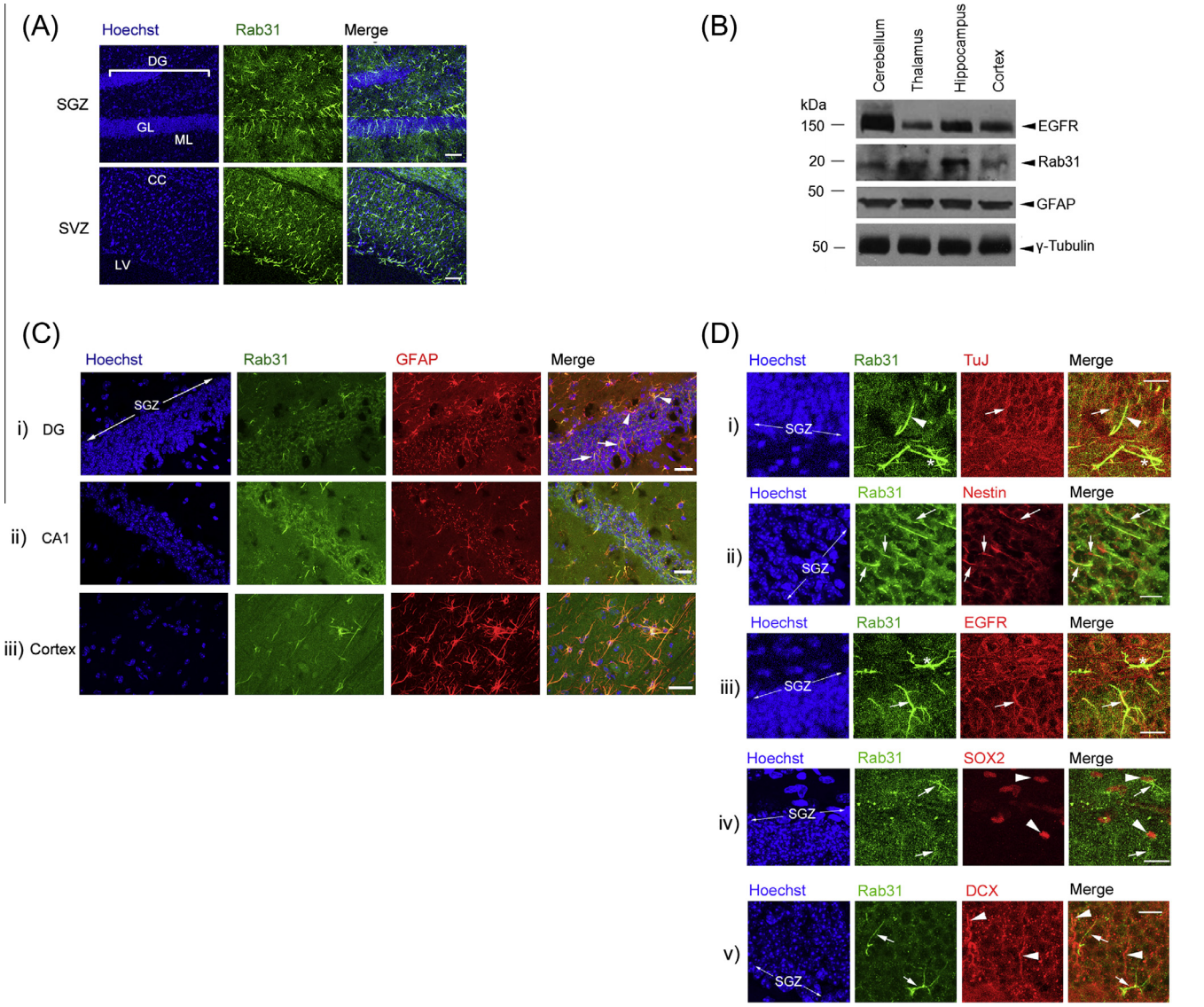

Fig. 1. Rab31 is found in the neurogenic zones of the adult rodent brain. (A) Adult mouse brain was perfused with 4% paraformaldehyde (PF), sectioned to 20 μm thickness on a cryostat and probed with Rab31 antibody (green). The subgranular zone (SGZ) and subventricular zone (SVZ) were identified by brain structural anatomy guided by the nuclear staining (Hoechst 33342, blue). In the upper row, the SGZ lies along the top border of the GL. In the lower row, the SVZ lies in the region between the LV and CC. DG: Dentate gyrus; GL: Granular layer; ML: Molecular layer; CC: Corpus callosum; LV: Lateral ventricle. Scale bar = 40 μm. (B) Various portions of the adult rat brain were dissected and lysed in extraction buffer. 100 μg of lysate was used for Western blot and probed for the various proteins indicated. Band densities were normalised against γ-tubulin. (C) Adult mouse brain was perfused with 4% PF, sectioned to 20 μm thickness on a cryostat, and probed with Rab31 (green) and GFAP (red) antibodies. DG (i), CA1 (ii), and cortical (iii) regions were identified by brain structural anatomy guided by the nuclear staining (Hoechst 33342, blue). The region of the SGZ is shown in the Hoechst-stained panel. Arrowheads indicate star-shaped cells typical of parenchymal astrocytes; arrows indicate radial astrocytes. Scale bar = 20 μm. (D) Adult mouse brain was perfused with 4% PF, sectioned to 20 μm thickness on a cryostat, and probed with Rab31 and various markers as indicated. Nuclei are marked by Hoechst 33342 (blue). The region of the SGZ is shown in the Hoechst-stained panel. *(i)*: Arrowhead points to a Rab31-positive radial astrocyte (green) extending into the granular layer. Arrow points to a TuJ-positive cell (red). Asterisk indicates a parenchymal astrocyte. *(ii)*: Arrows indicate some of the Rab31 (green) and nestin-positive (red) fibres. *(iii)*: Arrow points to a Rab31 (green) and EGFR-positive (red) radial astrocyte. Asterisk indicates a parenchymal astrocyte. *(iv)*: Arrows point to Rab31-positive fibres (green) originating from SOX2-positive (red) cells (arrowheads). *(v)*: Arrows point to Rab31-positive fibres (green), which do not colocalise with DCX-positive fibres of immature neurons (red, arrowheads). Scale bar = 20 μm.

and investigated if there was extraneous differentiation observed in Rab31-depleted NPCs. We found no morphological evidence that loss of Rab31 would itself induce cells to differentiate (Supp. Fig. 2B). This was corroborated by a Western blot which showed no decrease in Nestin and PCNA levels (Supp. Fig. 2C). The results suggest that manipulation of the levels of Rab31 does not have a significant impact on the undifferentiated state of NPCs.

We next investigated whether Rab31 influences the differentiation of NPCs. When induced to differentiate, there were significantly fewer GFAP-positive cells obtained in cells in which Rab31 was silenced (as indicated by the presence of GFP), compared to controls. We quantified this and found that Rab31-silenced cells have a reduced percentage of GFAP-positive cells obtained (Fig. 5A). There was no difference in the percentage of DCX-positive cells (data not shown). We then overexpressed Rab31 and induced the NPCs to differentiate (Fig. 5B). Overexpression of Rab31 (arrowheads) conversely enhanced the percentage of GFAP-positive astrocytes obtained. Taken together, our results suggest that Rab31 deficiency reduces, while overexpressing Rab31 increases NPC differentiation into GFAP-positive astrocytes (Fig. 5C). Rab31 may therefore be important for the differentiation of NPCs along the astroglia lineage.

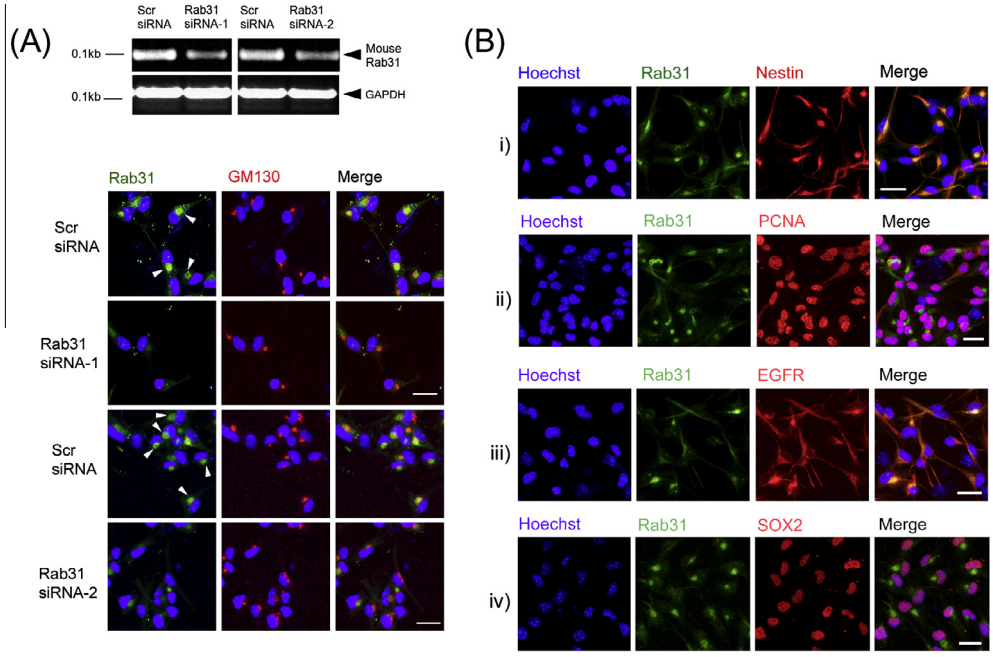

Fig. 2. Rab31 is expressed in undifferentiated neural progenitor cells (NPC). NPC were isolated from E15 mouse brain. (A) Undifferentiated cells were plated, and transfected with scrambled (Scr) or Rab31 siRNA as indicated. After 48 h cells were probed for knockdown of Rab31 by RT-PCR. Cells were also fixed with 4% PF and probed for Rab31 (green) and GM130 (red). The Western blot panel reflecting the effectiveness of the siRNA in silencing Rab31 is shown in Supp. Fig. 1. Arrowheads indicate examples of perinuclear Rab31 in the Scrambled (Scr) siRNA panels, which is not seen in the Rab31 siRNA panels. Nuclei are visualised with Hoechst 33342 (blue). Scale bar = 20 μm. (B) Undifferentiated cells were plated, fixed with 4% PF and probed for Rab31 (green) and the various markers indicated (red). Nuclei are visualised with Hoechst 33342 (blue). Scale bar = 20 μm.

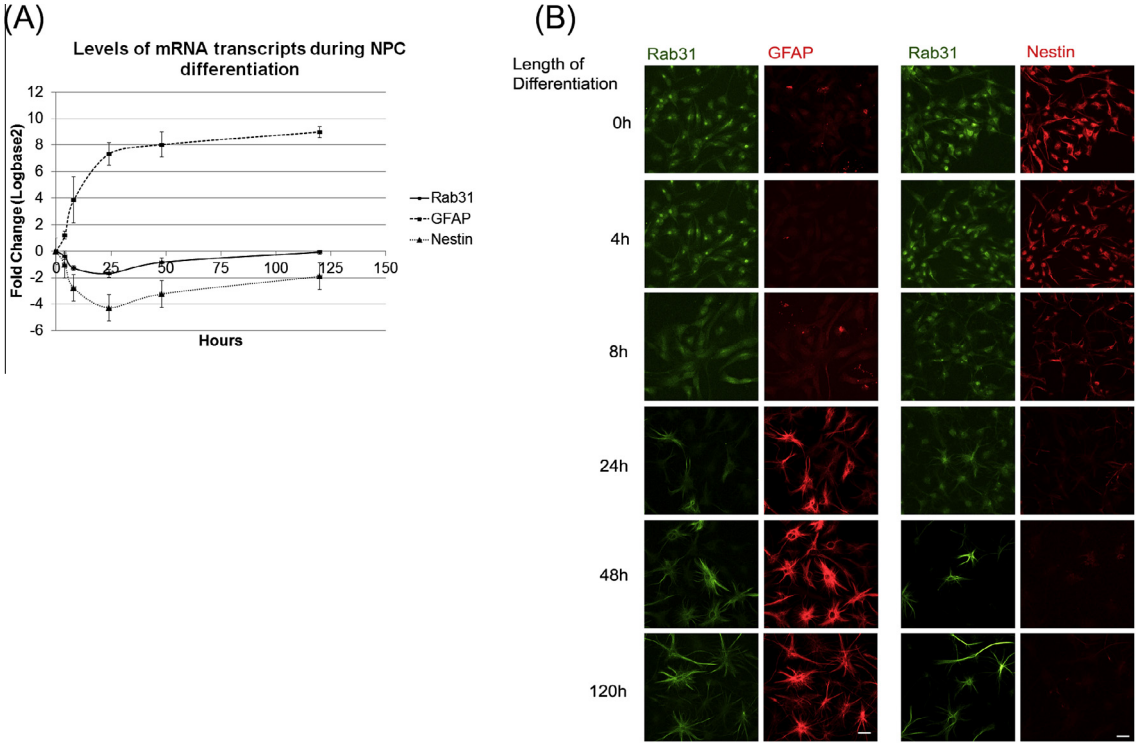

Fig. 3. Rab31 levels change in NPCs induced to differentiate into astrocytes. (A) Mouse NPCs were induced to differentiate in DMEM/F12 + 1% FBS and 10 ng/ml PDGFbb. Total mRNA was harvested from cells at various time points indicated. Quantitative real-time PCR was used to determine the changes in mRNA levels of Rab31, GFAP, and nestin, normalised to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and represented as fold changes compared to the 0 h time point. Three independent experiments were performed and data are shown as mean ± S.E.M. (B) Cells were fixed at various times indicated and probed for Rab31 (green) and GFAP or nestin (red). Scale bar = 20 μm. h = hours.

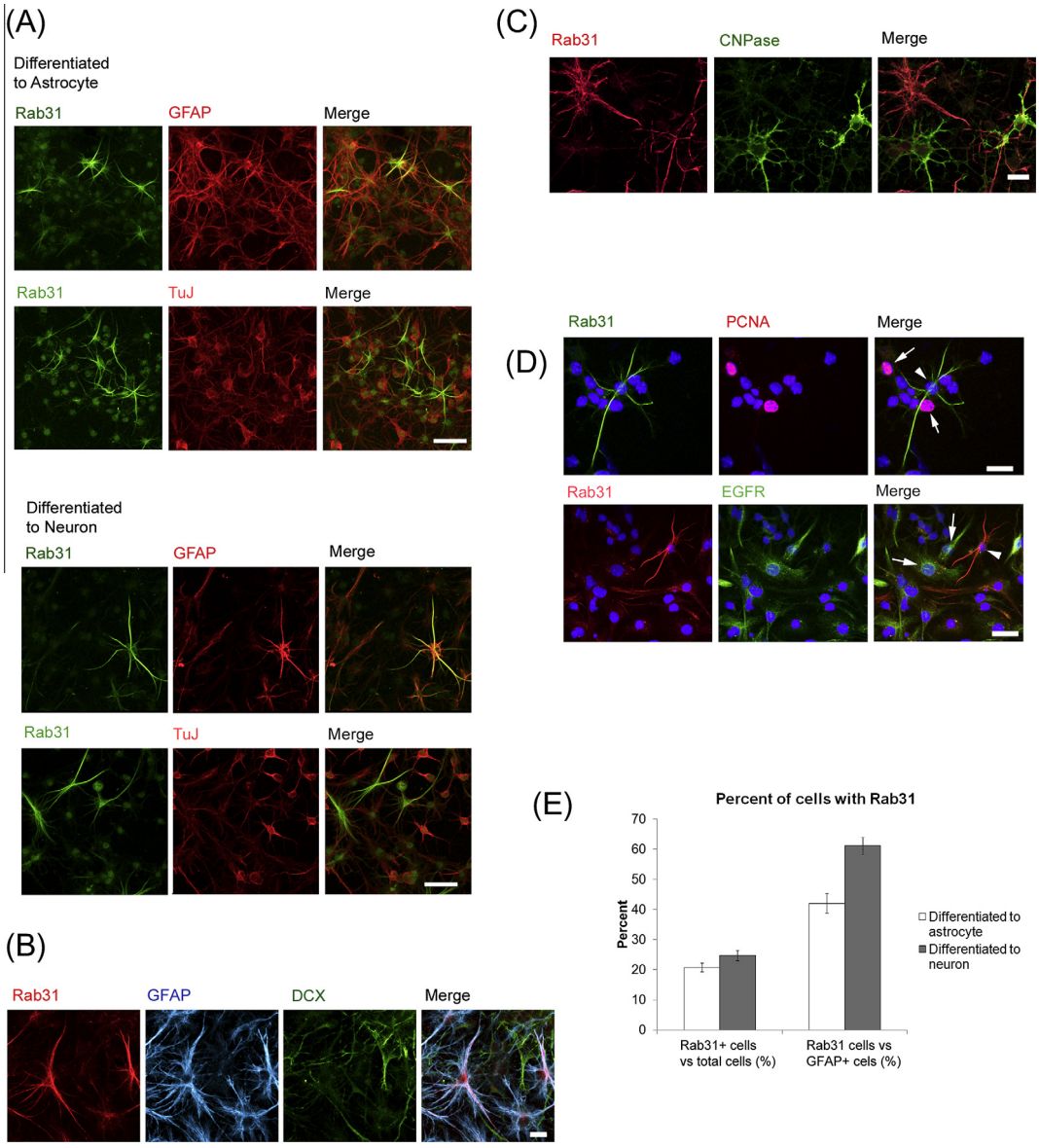

Fig. 4. Rab31 levels are elevated in a subset of GFAP-positive cells when NPCs are induced to differentiate. Mouse NPC were plated and induced to differentiate under various conditions for 5 days before fixation and immunofluorescence analysis. (A) Cells were probed for Rab31 (green) and GFAP or TuJ (red). Rab31 is highly expressed in a subset of GFAP-positive cells but not TuJ-positive cells, in both types of differentiation conditions. Scale bar = 50 μm. (B) Cells were probed for Rab31 (red), GFAP (pseudo-coloured blue) and DCX (green). Scale bar = 20 μm. (C) Cells were probed for Rab31 (red) and CNPase (green). Scale bar = 20 μm. (D) Cells were probed for Rab31 and various markers as indicated. Arrows indicate PCNA or EGFR positive cells; arrowhead points to a Rab31-positive cell. Nuclei were visualised with Hoechst 33342 (blue). Scale bar = 20 μm. (E) Cells were probed for Rab31 and GFAP. Number of Rab31-positive cells was quantified and presented graphically as a percentage of total cells and total GFAP-positive cells counted, respectively. At least 34 cells per experiment were analysed in 3 independent experiments, and data are shown as mean ± S.E.M.

4. Discussion

4.1. Role of Rab31 in NPCs

Our results suggest that Rab31 exerts a positive influence on the generation of astrocytes from neural progenitors, and has therefore a distinct physiological role to play in the mammalian brain. This, to our knowledge, is the first indication for a role of a Rab GTPase in astroglial differentiation.

How might Rab31 levels influence NPC cell fate? We have previously shown that Rab31 plays a role in the trafficking of ligand-bound EGFR [29]. Silencing of Rab31 delayed the degradation of EGFR, by hindering its entry into late endosomes, while overexpression of Rab31 enhanced the degradation. It is conceivable that the effect of Rab31 on the differentiation of NPCs is due to its role in EGFR trafficking.

At early stages, a low level of EGFR signalling was shown to be required for proliferation of neural progenitor cells [35]. Overexpression of EGFR at this stage causes NPCs to present the characteristics of differentiating astrocytes [36]. Our observation of the presence of Rab31 in NPCs (Fig. 2) may thus reflect their role in a fast turnover of ligand-bound EGFR, keeping EGFR signalling at a low level required for the proliferation of neural progenitor cells. While manipulating Rab31 levels did not affect the undifferentiated state of NPCs in our hands, this may simply be a result of other feedback or redundancy mechanisms in place. Because of the elongated nature of NPCs and the lack of antibodies suited for immunocytochemical observation of endocytic trafficking in this cell type,

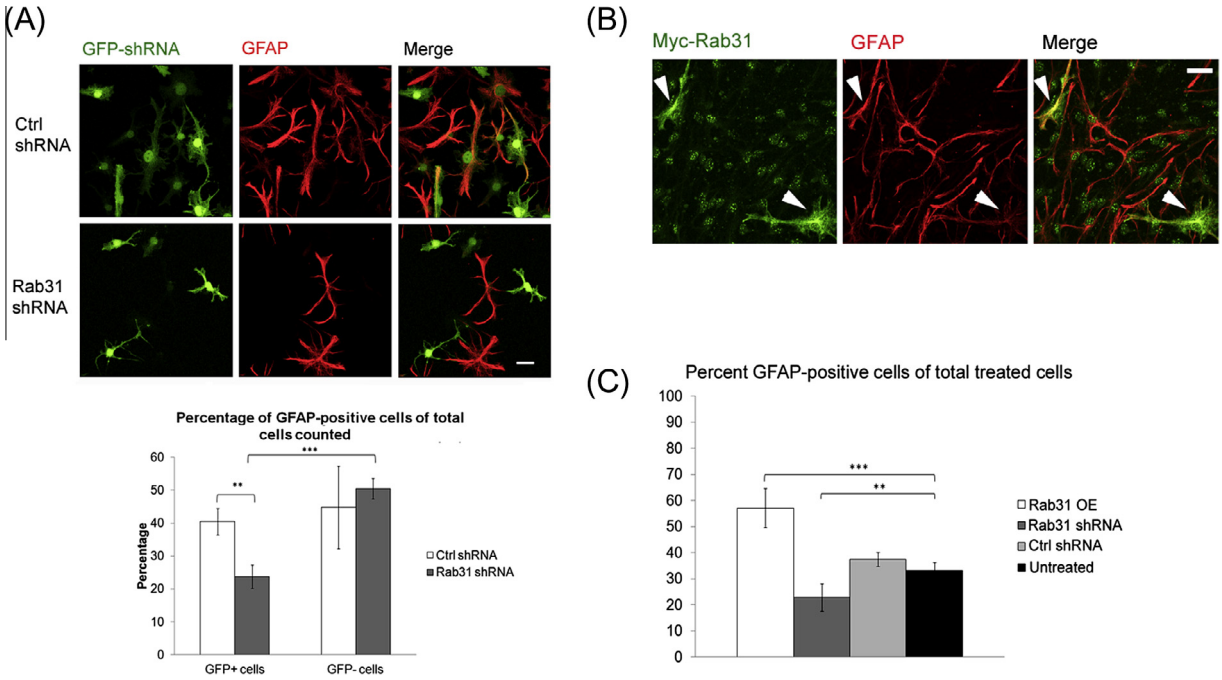

Fig. 5. Manipulations of Rab31 levels affect the number of GFAP-positive cells obtained when NPCs are induced to differentiate. Mouse NPCs were plated and cultured with medium containing GFP-Rab31 shRNA retroviruses. After 48 h, cells were induced to differentiate as indicated. (A) *Upper panel:* After 5 days cells were fixed in 4% PF and immunofluorescence staining for GFAP (red) was performed. Cells that have taken up the retroviral shRNA express GFP. Scale bar = 20 μm. *Lower panel:* Number of GFAP-positive cells was quantified and presented graphically as a percentage of total cells counted. At least 37 cells per experiment were analysed in 3 independent experiments, and data are shown as mean ± S.E.M. White bars represent counts from cells cultured with medium containing the control shRNA retroviruses (Ctrl), while shaded bars represent counts from cells cultured with Rab31 shRNA retroviruses. $^{**}P < 0.01$ and $^{***}P < 0.001$ by Student's *t*-test. (B) Mouse NPCs were plated and transfected with myc-labelled Rab31 (Myc-Rab31). After 48 h, cells were induced to differentiate. After 5 days cells were fixed in 4% PF and immunofluorescence staining for Myc-Rab31 (green, arrowheads) and GFAP (red) was performed. Scale bar = 20 μm. (C) NPCs were treated as indicated and induced to differentiate after 48 h. Cells were maintained in culture for 5 days before fixing in 4% PF and immunofluorescence staining for GFAP. Number of GFAP-positive cells was quantified and presented graphically as a percentage of total treated cells counted. Treated cells were identified by the Myc-Rab31 expression (for Rab31 overexpression (OE)) or the GFP expression (for shRNA-treated cells). At least 33 cells per experiment were analysed in 3 independent experiments, and data are shown as mean ± S.E.M. $^{**}P < 0.01$ and $^{***}P < 0.001$by Student's *t*-test.

we were unable, at this point, to fully explore the dynamic nature of Rab31-mediated EGFR trafficking in NPCs.

At later stages of embryonic development, a high level of EGFR signalling is required for differentiation to astrocytes [35]. It has been shown that EGFR and its ligands gradually increase in the developing CNS, coincident with gliogenesis [37,38]. During asymmetrical division of progenitor cells, daughter cells with high EGFR levels become astrocytes while those with low levels become oligodendrocytes [39]. In our observations, when NPCs are induced to differentiate, Rab31 shows a dip in levels initially (Fig. 3). This may aid in reducing the rate of degradation of ligand-bound EGFR, prolonging the effect of its signalling, to enable the differentiation to astrocytes.

A role in regulating the duration of EGFR signalling, however, cannot properly explain why Rab31 silencing would decrease (rather than increase) the eventual number of astrocytes obtained, and vice versa. One possible reason could be linked to a recent observation that EGF signalling, while enhancing the progression of radial glia to immature astrocytes, subsequently inhibits the progression from immature astrocytes to mature astrocytes (which express S100β along with GFAP) [12]. The increase in EGFR signalling caused by our Rab31 silencing studies might have resulted in a hindrance to the complete development of the astrocytes in culture, thus resulting in a decreased percentage of astrocytes that survive in culture.

We had attempted to further explore the idea of a Rab31-EGFR effect on astrocytic differentiation by withdrawing EGF from the culture medium. Because overexpression of Rab31 was shown to increase the rate of EGFR degradation, and therefore limit the extent of ligand-bound EGFR signalling [40], we postulated that a withdrawal of EGF from the media in which NPCs were cultured could crudely recapitulate the effects seen. Withdrawal of EGF, however, reduced the number of GFAP-positive astrocytes obtained (data not shown). One possible explanation for this is that EGF withdrawal is a poor mimic of the effect of overexpression of Rab31. Firstly, overexpression of Rab31 certainly would not completely abrogate EGFR signalling, but rather alters its strength and/or duration. Secondly, manipulation of Rab31 levels may alter subtle balances in EGFR trafficking or trigger feedback mechanisms that affect signalling. We should also note that besides EGF, a myriad of other factors are involved in signalling in NPCs, including fibroblast growth factor (FGF)-2, platelet derived growth factor (PDGF) [41], and transforming growth factor (TGF)α. While we have found that Rab31 does not participate in the trafficking of FGF receptor and PDGF receptor α (data not shown), TGFα also binds to EGFR and its signalling may therefore also be affected by Rab31.

4.2. Role of Rab31 in mature astrocytes

EGFR signalling is not only important for progenitor cells, it is also important for mature, differentiated astrocytes. It has been shown that EGFR signalling induces astrocytes to provide a permissive environment for neurite outgrowth in the developing CNS [42]. As high levels of Rab31 would enhance ligand-bound EGFR degradation and hence reduce the extent of EGFR signalling, this may be why we observed that a majority of astrocytes in our differentiating NPC culture do not display high levels of Rab31 (Fig. 4D). The reason for a subset of astrocytes expressing high levels of Rab31 in our differentiated NPC culture and adult mouse brain

cryosection remains to be understood. Given that EGFR signalling is very much reduced in mature astrocytes, the enrichment of Rab31 in a subset of the astrocytic population, even those outside of the neurogenic regions, may be reflective of a separate role of Rab31 from EGFR trafficking.

4.3. Implications and applications

It is speculated that NPCs have a large potential for CNS transplantation therapy. However, one problem frequently encountered is that astrogliosis is often triggered in an injured CNS environment, which results in eventual scar formation and inhibits axon or neuronal regeneration. Our results indicate that silencing Rab31 in NPCs can attenuate astroglia formation. It is therefore conceivable that manipulation of Rab31 levels in NPCs before transplantation into a site of traumatic CNS injury could aid in improving neuronal differentiation and neurite growth. Moreover, it has been shown that blocking EGFR activation in astrocytes is beneficial for neuronal survival in cases of traumatic CNS injury, as it minimises the activation of reactive astrocytes [35]. Since Rab31 has been associated with EGFR trafficking, manipulation of Rab31 levels in mature astrocytes might also prove beneficial. Because of the multivariate effects of EGFR signalling in neurodevelopment as well as tumour progression, manipulation of EGFR signalling in therapeutic strategies must be tempered with caution. Interestingly, Rab31 has been shown to be of value as a biomarker in Glioblastoma multiforme (GBM), a form a brain cancer caused by malignant glioma [43], and was recently found to be upregulated in GBM [44]. NPCs are believed to be the cell of origin of GBM tumour stem cells. Therefore, a better understanding of how EGFR signalling is regulated by Rab31 would be critical. To this end, studying the role of Rab31 therefore also becomes important.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.06.060.

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